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REVIEW ARTICLE

DNA vaccine-mediated innate immune response triggered by PRRs in teleosts

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Abstract Aquacultured fish are threatened by many pathogens, often with serious consequences. Vaccination is one of the most effective tools for enhancing host immunity and protecting fish from infections. Vaccination with DNA vaccine is based on the administration of the gene encoding a vaccine antigen. Several effective DNA vaccines that encode viral or bacterial antigenic proteins have already been shown to be effective for cultured fish. This review summarizes current knowledge on fish DNA vaccines, and the mechanism of interaction between the DNA vaccines and host immunity, especially focusing on the enhancement of innate immunity mediated through direct-recognition of DNA vaccine by pattern recognition receptors (PRRs). To date, numerous fish PRR genes have been identified, and the primordial functions of PRRs involved in the innate immune response to viral and bacterial nucleic acids have been increasingly clarified. The evolutionary conservations and divergences in the PRR mechanisms of teleosts and mammals are focused on their molecular features and the recognition of DNA vaccine mediated by TANK binding kinase 1. In addition, the mechanism of type I interferon production in teleosts, which is enhanced after the recognition of cytosolic nucleic acids and current topics on DNA sensing by PRRs are also introduced.

Keywords DNA vaccine · Innate immunity · Pattern recognition receptors (PRRs) · Nucleic acid sensing · Type I interferon (IFN)

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Immune response activated through DNA vaccine

DNA vaccine against fish pathogens

Live, weakened, or killed bacterial pathogens, which were the first generation vaccines, are composed of whole pathogens. Bacterins are killed pathogenic bacteria that induce the humoral immune response such as the production of neutralizing antibodies. Vaccines made from live (or attenuated) pathogens can stimulate both cellular and humoral immune responses. Both types of vaccine are currently used in aquaculture [1, 2]. Second generation vaccines consist of antigenic subunits from the pathogen. These vaccines, called subunit vaccines, elicit humoral immune responses, such as production of neutralizing antibodies, but not cellular immune responses. Third generation vaccines or DNA vaccines are directly inoculated plasmid DNAs that encode a specific protective antigen gene under the control of a eukaryotic promoter. In mammals, once the plasmid DNA is administered in vivo, the encoded antigen is thought to be expressed in the host cells. At this point, antigen-presenting cells, such as dendritic cells, take up the antigen, process it and elicit the production of neutralizing antibodies, helper

Table 1 DNA vaccine studies on viral and bacterial pathogens in fish

Pathogens	Antigen gene inserted	Host	Route	References
RNA viruses				
Atlantic halibut nodavirus (AHNV)	Coat protein	Turbot (*Scophthalmus maximus*)	Intramuscular (i.m.)	[118]
Infectious hematopoietic necrosis virus (IHNV)	Glycoprotein	Rainbow trout (*Oncorhynchus mykiss*)	i.m.	[4]
	Nucleocapsid protein	Rainbow trout	i.m.	[4]
Infectious pancreatic necrosis virus (IPNV)	Polyprotein plus VP2	Atlantic salmon (*Salmo salar*)	i.m.	[119]
	VP2	Rainbow trout	Oral (with microspheres)	[11]
Infectious salmon anaemia virus (ISAV)	Hemagglutinin-esterase	Atlantic salmon	i.m.	[120]
	Nucleoprotein	Atlantic salmon	i.m.	[120]
Hirame rhabdovirus (HIRRV)	Glycoprotein	Japanese flounder	i.m.	[10, 22]
	Nucleocapsid protein	Japanese flounder	i.m.	[22]
Spring viraemia of carp virus (SVCV)	Glycoprotein	Common carp	i.m.	[13]
		Koi (*Cyprinus carpio haematopterus*)	i.m.	[121]
Viral haemorrhagic septicaemia virus (VHSV)	Glycoprotein	Rainbow trout	i.m.	[5, 122]
		Japanese flounder	i.m.	[20]
VHSV and IHNV (cocktail)	Glycoproteins	Rainbow trout	i.m.	[123]
DNA viruses				
Channel catfish herpesvirus (CCHV)	ORF 59 plus ORF 6	Channel catfish (*Ictalurus punctatus*)	i.m.	[124]
Lymphocystis disease virus (LCDV)	Major capsid protein	Japanese flounder	Oral (with PLGA nanoparticles)	[125]
Koi herpesvirus (KHV)	ORF25 (glycosilated protein)	Koi	i.m.	[126]
Red seabream iridovirus (RSIV)	Major capsid protein	Red sea bream (*Pagrus major*)	i.m.	[12]
Bacteria				
Aeromonas veronii	Omp38 and Omp48	Spotted sand bass (*Paralabrax maculatofasciatus*)	i.m.	[127]
Edwardsiella tarda	Eta6	Japanese flounder	i.m.	[14]
	D15-like surface antigen	Japanese flounder	i.m.	[16]
	Eta2	Japanese flounder	i.m.	[15]
Flavobacterium psychrophilum	Hsp60 and 70	Rainbow trout	i.m.	[128]
Mycobacterium marinum	Ag85A	Hybrid striped bass (*Morone saxatilis* x *M. chrysops*)	i.m.	[17, 18]
Streptococcus agalactiae	Surface immunogenic protein	Nile tilapia (*Oreochromis niloticus*)	Oral (with recombinant bacteria)	[129]
S. iniae	Secretory antigen Sia10	Turbot	i.m.	[130]
Vibrio alginolyticus	OmpW	Crimson snapper (*Lutjanus erythropterus*)	i.m.	[131]
V. anguillarum	38-kDa major outer membrane prion protein	Asian seabass (*Lates calcarifer*)	i.m.	[132]
V. harveyi	DegQ and Vhp1	Japanese flounder	i.m.	[133]
S. iniae and *V. anguillarum*	Sia10 and OmpU	Japanese flounder	i.m.	[134]

T cells, and cytotoxic T lymphocytes (CTLs). This immune cascade results in the elimination of intracellular pathogens, and is a unique feature of DNA vaccines. Many studies have investigated DNA vaccines against fish pathogens, especially viruses and intracellular bacteria [2, 3] (Table 1). DNA vaccines against rhabdoviruses, such as infectious hematopoitic necrosis virus (IHNV), viral haemorrhagic septicaemia virus (VHSV), have been most successful [4, 5]. A DNA vaccine against IHNV has been approved for use in Canada [6]. The glycoprotein (G-protein) genes are highly immunogenic and are often utilized to develop DNA vaccines against rhabdoviruses. All that is needed is an

intramuscular (i.m.) injection of purified plasmid DNA. In rainbow trout fingerlings, nanogram levels of plasmid DNA are enough to induce protective immunity against IHNV and VHSV [7–9]. DNA vaccines have been found to be effective against hirame rhabdovirus (HIRRV) [10], infectious pancreatic necrosis virus (IPNV) [11], red seabream iridovirus (RSIV) [12], and spring viraemia of carp virus (SVCV) [13]. They may also be useful against intracellular bacterial pathogens, including *Edwardsiella tarda* [5, 14–16] and *Mycobacterium marinum* [17, 18].

Transcriptomic analysis of DNA vaccine-inoculated fish and the expected immune responses

We have previously analyzed the expression of Japanese flounder *Paralichthys olivaceus* genes in DNA vaccine-inoculated fish using DNA microarray [19–23].

The genes involving lymphocyte activation, such as CD20, CD40 and B lymphocyte cell adhesion molecules were up-regulated at 1 and 3 days post-immunization with VHSV G-protein gene [20, 21]. Homologs of mammalian T cell activation-related molecules (such as cytohesin-1, CXCR3, CARD11/CARMA1, gp96, CaMKII, DAP10, DC-SIGN, PA28α and α2m) were also up-regulated in Japanese flounder immunized with HIRRV G-protein gene [23]. Up-regulation of these genes may suggest that HIRRV G-protein gene induces B cell activity and antigen-specific humoral immunity. Inoculation of fish with G-protein genes of VHSV and IHNV induced the production of neutralizing antibodies which may have helped to protect against these viruses [24–26]. Hence, B cells activated by DNA vaccination against VHSV G-protein gene might play an important role in the immune response [27].

On the other hand, DNA vaccination against rhabdovirus provided high levels of specific protection without producing detectable amounts of neutralizing antibodies [5, 28]. A cDNA microarray analysis of Japanese flounder immunized with DNA vaccines encoding rhabdovirus G-proteins showed up-regulation of the genes that were involved in cellular immune responses, such as the CD8 α chain gene [20–23]. The role of cellular immunity in the DNA vaccine-inoculated fish has not yet been clarified. However, Somamoto et al. [29, 30] reported a specific cell-mediated immune response of crucian carp lymphocytes to the MHC-matched cells infected with crucian carp hematopoietic necrosis virus. Further, $CD8^+$ cytotoxic T cells (CTLs) were considered to be involved in the antiviral adaptive immunity of the carp [30]. Taken together, $CD8^+$ CTLs of teleosts may also have a role in the specific protection provided by DNA vaccination.

In addition to inducing antigen-specific immune responses, DNA vaccination also induces non-specific innate immune responses. Vaccination of rainbow trout with the G-protein gene from IHNV induced early antiviral defense against a VHSV challenge, suggesting that DNA vaccination is also involved in the activation of the innate immune system [31]. Using a Japanese flounder cDNA microarray, we detected an immediate up-regulation of non-specific immune response genes, including NK Kupffer cell receptors, MIP1-a, and antiviral Mx1 protein (Mx1), following administration of VHSV G-protein genes [20, 21]. Furthermore, the interferon-stimulated gene 15 kDa (ISG15), ISG56, and Mx1 were strongly induced after the vaccination with the HIRRV G-protein gene [22, 23]. Mx1, ISG15, and ISG56 are known as type I interferon (IFN)-inducible genes, which inhibit viral replication and protein synthesis [32–36]. Interestingly, these type I IFN-inducible genes were induced by the protective HIRRV G-protein immunization but not by the inefficient nucleocapsid (N) protein immunization [22]. Similarly, type I IFN-related genes were up-regulated at systemic sites (e.g., gills, kidney, and spleen) by the IHNV G-protein gene in rainbow trout. Taken together, these findings indicate that induction of the type I IFN system across multiple tissues may be one of the major functions of early anti-viral innate immunity in DNA-vaccinated fish [37].

Although the innate immune system provides an early defense against infection, it has been considered to act as a temporary system until adaptive immune responses can be triggered. However, from the recent studies in mammals, it is currently believed that activation of innate immunity is a prerequisite for induction of acquired immunity [38]. For a DNA vaccine to induce an effective immune response against fish pathogenic viruses, there must be both a type I IFN-mediated innate immune response and an adaptive immune response (Fig. 1). Type-I IFN systems in teleosts are distinct from those in mammals, because teleost species have had a long and complex genome history and have developed a specific adaptation to the aquatic environment [39]. Hence, uncovering the mechanism for induction of the type I IFN system following DNA vaccination of teleosts is a subject of great interest. In the next section, we summarize the present knowledge regarding the molecules involved in recognition of a DNA vaccine and activation of IFNs in teleosts.

The immune responses activated by nucleic acid recognition

Pattern recognition receptors (PRRs) involved in nucleic acid recognition in teleosts

In mammals, the innate immune response is initiated through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors

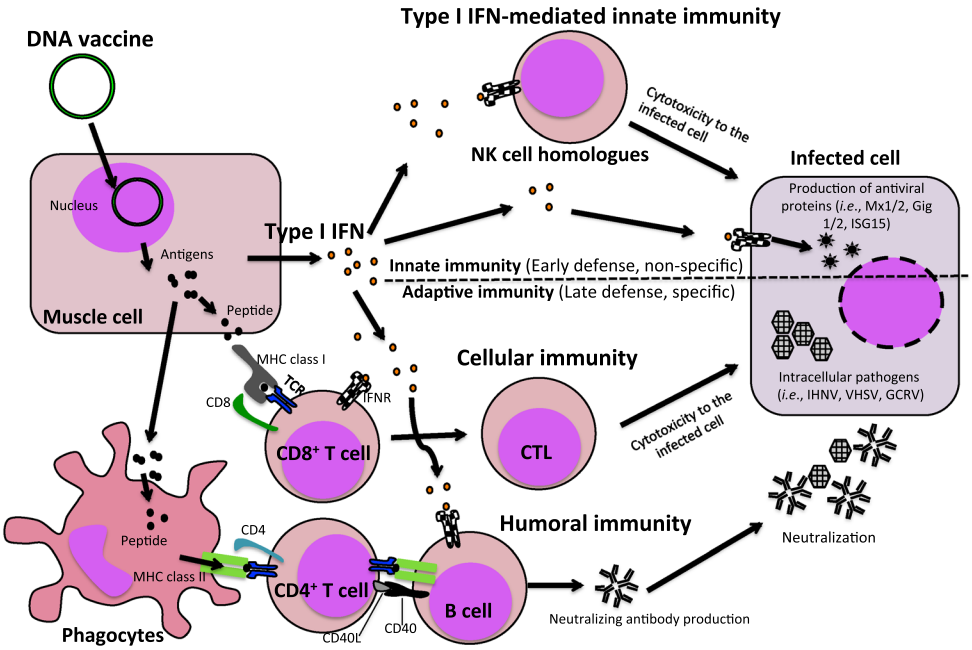

Fig. 1 Schematic overview of type 1 IFN-mediated immune responses in DNA vaccine inoculated fish. Type I IFN activates both innate and adaptive immunity to modulate an effective system to eliminate intracellular pathogens. *CTL* cytotoxic T lymphocyte, *IFN* interferon, *IFNR* interferon receptor, *MHC* major histocompatibility complex, *TCR* T-cell receptor

(PRRs). The signaling PRRs include the Toll-like receptors (TLRs), retinoic acid inducible gene (RIG)-I-like receptors (RLRs), and nucleotide-oligomerization domain (NOD)-like receptors (NLRs). The DNA of the vaccine is directly recognized by some of these receptors, which then trigger innate immune responses [2, 3, 40]. These receptors include CpG DNA sensors (e.g., TLR9), B-form DNA sensors (e.g., Z-DNA binding protein-1; ZBP), and inflammasomes. ZBP is also known as DAI (DNA-dependent activator of IFN-regulatory factors). Inflammasomes include NACHT-, leucine-rich repeat (LRR)-, and pyrin domain (PYD)-containing proteins (NALP3). For example, DNA vaccines containing human-specific CpG motifs induced the maturation of human monocytes, suggesting that improvements to plasmid DNA for innate immune signaling activation are important for the enhancement of immunogenicity and induction of optimal immune responses [41, 42]. In teleosts, six TLRs and three RLRs have been demonstrated to recognize nucleic acids (Table 2), while NLR members which are responsible for exogenous nucleotide recognition have not been identified except for AIM2 described in the section after next, "Other current topics on cytosolic DNA sensors (CDSs)". In teleosts, PRR-associated sensing of exogenous nucleotides has been shown to lead to antiviral responses via the production of type I IFN and other cytokines. Hence, exogenous nucleic acids (i.e., DNA vaccines, viral RNA, and bacterial DNA) and their analogs can be used as vaccine adjuvants for aquaculture [43]. In this section, therefore, we summarize the nucleotide-sensing PRRs and their cascades in teleosts.

Nucleic acid-sensing TLRs in teleosts

TLRs are homologues of the *Drosophila* Toll receptor. The latter was initially identified as a receptor essential for dorsoventral polarity during development, and was shown to participate in the innate immune responses against fungal infections [44]. In mammals, TLRs are widely distributed on the cell surface and on the endosomal membrane of immune cells, including macrophages, dendritic cells (DCs), B cells, and specific types of T cells. Both fibroblasts and epithelial cells, which are non-immune cells, also express TLRs [45, 46]. TLRs are type-1 trans-membrane glycoproteins, which comprise an N-terminal extracellular leucine-rich repeat (LRR) domain and a C-terminal intracellular Toll/IL-1 receptor (TIR) domain. The LRR domain is responsible for the recognition of ligands specific to a particular PAMP [47]. These structural characteristics are conserved in vertebrate TLRs, except the soluble form of TLRs (e.g., TLR5S in teleosts) [48, 49]. Several teleost TLRs (TLR3, -7, -8, -9, -21 and -22) may be able to sense nucleic acids [50].

TLR3 is an endosomal membrane receptor that recognizes pathogen-derived double-stranded RNAs (dsRNAs), such as dsRNA virus genomes. The dsRNA analog poly I:C has often been used as an antiviral immune response mediator in teleosts. Injection of poly I:C into Atlantic salmon resulted in the production of type I IFN and expression of antiviral Mx gene in immune-related tissues [51]. In addition, in human embryonic kidney (HEK) 293 cells expressing Japanese puffer TLR3 and rainbow trout gonadal (RTG)-2 cells, poly I:C stimulation was found to activate the IFN gene promoter [52].

Table 2 Research reports on nucleic acid sensing-PRRs in teleosts which demonstrated the functional involvement of the receptors to type I IFN and antiviral activity

PRRs	Ligands	Fish	Functional characteristics demonstrated in the report	References
TLRs				
TLR3	dsRNA, poly I:C	Japanese flounder	Poly I:C stimulation of JfTLR3-overexpressing cells significantly induced IFN-inducible gene and NF-κB reporter activity	[135]
		Rare minnow (*Gobiocypris rarus*)	TLR3-mediated Mx expression was confirmed by using RNAi and transgenic techniques	[136]
		Japanese pufferfish	TLR3 resides in endoplasmic reticulum and recognizes relatively short-sized dsRNA	[52]
TLR7	ssRNA, imidazoquinoline	Atlantic salmon	TLR7 gene expression was to type I IFN and IFNγ treatment in primary head kidney cells. IFNb and IFNc are the main IFNs induced through the TLR7 pathway because R848, an ssRNA analog, induced high transcript levels of IFNb and IFNc in the head kidney and spleen	[137, 138]
		Rainbow trout	Rainbow trout anterior kidney leukocytes produced elevated levels of pro-inflammatory and type I IFN cytokine mRNA in response to stimulation with the human TLR7/8 agonist R848	[139]
TLR8	ssRNA, imidazoquinoline	Atlantic salmon	Promoter analysis predicted several transcription factor binding sites in the TLR8a1/2 and TLR8b1 5′ flanking regions, namely C/EBP, AP-1, STAT, NFκB, and IRF family, suggesting cytokine regulation of the genes. TLR8a1 gene expression was sensitive to type I IFN and IFNγ treatment in salmon head kidney-1 cells	[137]
		Rainbow trout	Two TLR8 genes exist (TLR8a1 and TLR8a2) in rainbow trout. Rainbow trout anterior kidney leukocytes produced elevated levels of pro-inflammatory and type I IFN cytokine mRNA in response to stimulation with the human TLR7/8 agonist R848	[139]
TLR9	CpG-ODN	Atlantic salmon	Atlantic salmon TLR9 has been found to interact with synthetic oligonucleotides via a CpG-independent but a pH-dependent mechanism. When overexpressed in salmonid cell lines, the salmon TLR9 spontaneously activates ISRE-containing promoters of genes involved in the IFN response	[140, 141]
		Japanese flounder	The flounder tumor necrosis factor (TNF) gene promoter was activated in TLR9-transformed hirame natural embryo (HINAE) cells upon stimulation with synthesized CpG oligodeoxynucleotide (ODN). When the expression of the flounder TLR9 was knocked down in vivo by small interfering RNA, CpG ODN-mediated immune response and antiviral activity were significantly blocked	[59, 60]
TLR21	CpG-ODN	Zebrafish	Results from cell-based activation assays indicate that zebrafish TLR21 is functional, responding to CpG-ODN but not to other TLR ligands. TLR21 responded preferentially to CpG-ODN with GTCGTT motifs. TLR9 and TLR21 of zebrafish cooperatively mediate the antimicrobial activities of CpG-ODN	[66]
TLR22	Long-sized dsRNA	Japanese pufferfish	TLR22 recognizes long-sized dsRNA on the cell surface. When fish cells expressing TLR22 are exposed to dsRNA or aquatic dsRNA viruses, cells induce IFN responses to acquire resistance to virus infection	[52]

Table 2 continued

PRRs	Ligands	Fish	Functional characteristics demonstrated in the report	References
RLRs				
RIG-I	Viral RNA, 5′ppp-dsRNA	EPC cells	N-terminal of RIG-I led to a strong induction of both IFN and ISGs, conferring on fish cells protection against VHS infection	[142]
		Grass carp	Overexpression of RIG-I decreased the viral titer of GCRV in CIK cells. IFN production was significantly increased in RIG-1 gene transfected cells following GCRV infection	[86]
MDA5	Viral RNA, poly I:C	Japanese flounder	HINAE cells overexpressing MDA5 showed a lower cytopathic effect (CPE) against VHSV, hirame rhabdovirus (HIRRV) and infectious pancreatic necrosis virus (IPNV) infection	[70]
		Zebrafish	Gene expression of zebrafish MDA5 genes are induced by SVCV and *Edwardsiella tarda* infection. Overexpression of MDA5 genes in fish cells resulted in significant induction of type I IFN promoter activity and enabled the protection of transfected cells against SVCV infection	[143]
LGP2	Viral RNA, poly I:C	Japanese flounder	The expression of LGP2 mRNA increased after VHSV infection and poly I:C stimulation. Type I IFN and IFN-inducible genes (Mx and ISG15) in HINAE cells overexpressing LGP2 were increased by poly I:C and viral infections. LGP2 transcriptional control is crucially involved in regulation by interferon regulatory factor (IRF) 3	[69, 144]
		Rainbow trout	LGP2 were constitutively produced in fibroblast and macrophage cell lines and upregulated by poly I:C, recombinant IFN, or infection by RNA viruses. Overexpression of MDA5 and LGP2 resulted in significant accumulation of Mx transcripts in cultured cells	[145]

TLR7, -8 and -9 are also expressed on the endosome membranes. Mammalian TLR7 and -8 recognize synthetic antiviral imidazoquinoline compounds, which mimic single-stranded RNA (ssRNA), such as bacterial RNA and the RNA viral genomes [46, 53–55]. Imidazoquinolines, such as S-27609 and R848, are also efficient immune modulators in teleosts [56–58]. R848 stimulation was found to up-regulate the genes for IFN-α1, IL-1β, IL-8, and two types of tumor necrosis factor in rainbow trout leukocytes [56]. In salmon leukocytes, IFN-β genes were strongly up-regulated by S-27609, while IFN-α genes were up-regulated by poly I:C [58]. Hence, it is likely that salmon TLR7 and -8 are responsible for recognizing ssRNA to activate signaling pathways to produce IFN-β.

TLR9 recognizes the unmethylated CpG DNA motifs present in viral and bacterial genomes, and initiates immune responses through the production of inflammatory cytokines including type I IFN [46]. CpG motifs are also present in the plasmid backbone, and thus act as an intrinsic "built-in adjuvant" for DNA vaccines [42]. CpG oligodeoxynucleotides (ODNs), which mimic unmethylated CpG DNA, have been used to examine the role of teleost TLR9. In a transgenic Japanese flounder cell line expressing the TLR9 gene, CpG ODN stimulation was found to activate the tumor necrosis factor (TNF)-α gene promoter [59]. In flounder, knockdown of TLR9 by a small interfering RNA impaired CpG ODN-mediated immune response and antiviral activity [60]. These studies suggest that teleost TLR9 directly recognizes CpG-ODN. Among many kinds of CpG ODNs, CpG ODN 1681 induced the highest level of IFN-like activity in leukocytes of Atlantic salmon *Salmo salar* [61]. This IFN-like activity may be mediated by type I IFN and Mx protein, because these molecules were produced from Atlantic salmon leukocytes following stimulation with CpG ODN 1681 [62].

Mammals do not appear to have homologs of TLR21 and -22 [63]. Chicken TLR21 was suggested to be a CpG ODN receptor [64]. As the TLR21 genes of teleosts and chicken are similar, teleost TLR21 is also considered to be a CpG motif receptor [63, 65]. In fact, zebrafish *Danio rerio* TLR21 responded preferentially to CpG-ODN with GTCGTT motifs to exhibit antimicrobial activity [66]. Japanese puffer TLR22 was found to recognize longer dsRNAs on the cell surface, whereas tiger puffer TLR3 was found to recognize shorter dsRNAs in the endoplasmic reticulum [52]. Production of IFN was significantly up-regulated

when Japanese puffer *Takifugu rubripes* TLR22-expressing RTG-2 cells were exposed to dsRNA genome of IPNV, and the stimulated cells showed an increased resistance to IPNV infection [52].

Retinoic acid inducible gene (RIG)-I-like receptors (RLRs) in teleosts

The RLR family consists of three members: RIG-I which is also called DEAD box poly peptide 58 (DDX58); melanoma differentiation-associated gene 5 (MDA5), which is also called IFN induced with helicase C domain 1 (IFIN1); and laboratory of genetics and physiology 2 (LGP2), which is also called DExH box polypeptide 58 (DHX58) [46, 67, 68]. Teleost and mammalian RLRs have a number of structural similarities [65]. In teleosts, the RIG-I, MDA5 and LGP2 genes are expressed in most tissues and cell lines [65, 69–71]. The expression levels of the fish RLR mRNAs were increased in various tissues upon infection by several viruses, such as SVCV, grass carp *Ctenopharyngodon idellus* reovirus (GCRV) and IPNV [72–79].

In mammals, RLRs sense cytoplasmic viral ssRNAs and dsRNAs. Mammalian RIG-I is capable of recognizing 5′-triphosphate (5′ppp)-capped ssRNA and dsRNA (both capped and uncapped) [80–82], including poly I:C and viral dsRNAs generated by viral replication [82–84]. In contrast, MDA5 senses only long dsRNA containing both poly I:C and viral RNA [83]. LGP2 recognizes dsRNAs and 5′ppp-ssRNA [85].

In teleosts, overexpression of grass carp RIG-I enhanced type I IFN gene expression, and decreased the titer of grass carp reovirus (GCRV) to about the titer in grass carp kidney cells [86]. Further, the gene expression level of Mx2 was significantly higher in the grass carp RIG-I-transfected cells following GCRV infection or poly I:C stimulation than in the normal cells [86]. GCRV-induced gene 1 (Gig1), which is unique to fish species, was significantly induced by intracellular poly I:C through the IFN signaling pathway triggered by RIG-I-like receptors. Overexpression of the Gig1 gene in a crucian carp *Carassius auratus* blastulae embryonic (CAB) cell line resulted in a significant reduction of GCRV titers [87]. Similarly, in vitro antiviral activity against SVCV was potentiated in Gig2 (Drel) gene-transfected epithelioma papulosum cyprinid (EPC) cells [88]. Rapid production of Gig protein in response to poly I:C transfection in CAB cells were determined by immunofluorescence microscopy. The rapid and abundant expression helps Gig1 to quickly exert its antiviral effects when host cells respond to virus infection [87]. These finding suggest that the RIG-I system also plays a crucial role in antiviral activity in teleosts. We also demonstrated the involvement of RLRs in virus recognition and antiviral response in Japanese flounder. Hirame (Japanese flounder) natural embryo (HINAE) cells overexpressing MDA5 showed a decreased cytopathic effect (CPE) against VHSV, HIRRV and IPNV infection. When infected with VHSV, MDA5-overexpressing HINAE cells had significantly lower virus titer than normal HINAE cells [70]. Similarly, the CPEs of the viruses were delayed by the overexpression of Japanese flounder LGP2 in HINAE cells. Furthermore, mRNA expression levels of type I IFN and IFN-inducible genes (Mx and ISG15) in the LGP2-overexpressing cells were increased by poly I:C stimulation and viral infections [69]. These results suggest that teleost RLRs have roles in the recognition of ssRNA and dsRNA to induce the production of type I IFN and IFN-related antiviral proteins.

Signaling cascades for type I IFN production result from the nucleic acid-sensing PRRs in teleosts

The signaling pathways of TLRs are mediated by selective usage of adaptor molecules, MyD88, TRIF, TIRAP and TRAM [38].

On poly I:C-stimulated fish cells, Japanese puffer fish TLR3 and -22 recruit the TRIF adaptor protein (synonymous with Toll-IL-1-receptor-containing adaptor molecule-1; TICAM-1), which in turn moves from the TLR to a cytoplasmic signalosome region [52]. Thus, the TRIF is thought to act as a shuttling platform for IFN signaling via fish TLR3 and -22 [52]. In addition, the luciferase reporter assays have demonstrated that TRIFs of zebrafish and pufferfish activate type I IFNs [52, 89, 90].

Mammalian TLR7, -8, and -9-dependent production of type I IFN requires direct interaction of MyD88 and IRF7 [38]. In teleosts, although the TLR7 and -8 genes have also been identified, their roles and associated signaling molecules have not been elucidated. Mammalian TLR9 interacts with the TIR domain of MyD88 through a BB loop in the box 2 region of its cytosolic TIR domain, and this box region is also conserved in zebrafish TLR9 [66]. Mutation of residues in the box 2 abrogated activation of zebrafish TLR9 by CpG-ODNs [66]. Hence, zebrafish TLR9 is structurally and functionally similar to the mammalian TLR9, suggesting that zebrafish TLR9 relates to the MyD88-dependent signaling pathway. Cytosolic RLR-mediated signaling pathway also induces the production of type-I IFN in teleosts as mentioned above. The details of TANK-binding kinase 1 mediated type-I IFN production pathways are introduced in the next section.

The key kinase for DNA recognition, TANK-binding kinase 1 (TBK1)

In the recognition of double stranded DNA (dsDNA) in mammals, DNA vaccine-induced immunogenicity is

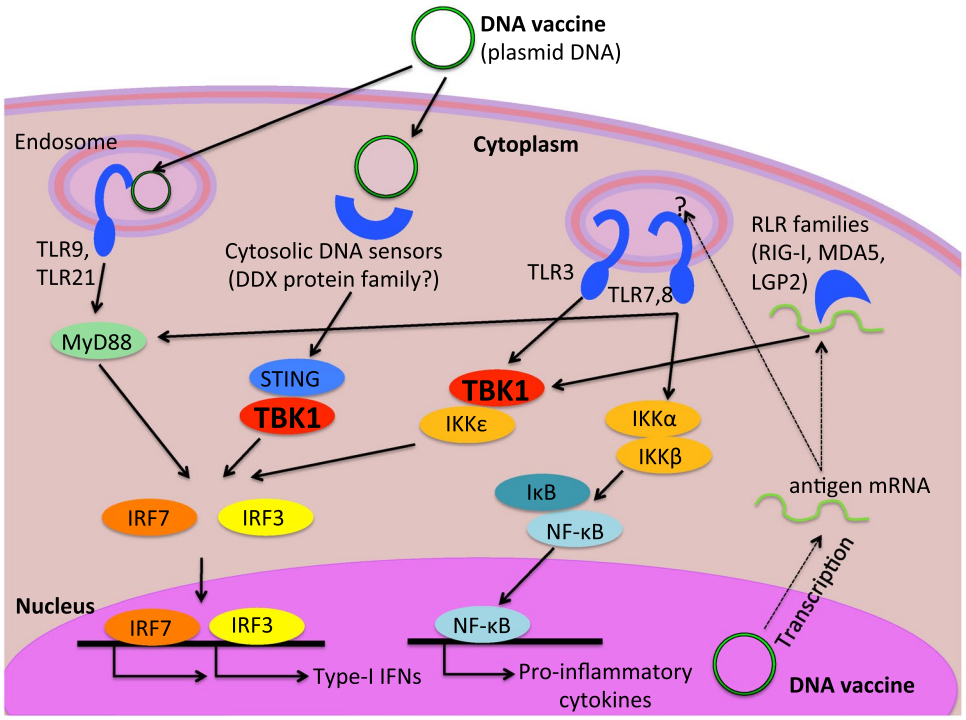

Fig. 2 Schematic diagram of the expected intracellular signaling pathways following DNA vaccination in fish. TLR9 and -21 recognize CpG motifs of DNA vaccine in the endosome, and then the MyD88-dependent pathway is activated. Cytosolic DNA sensors detect DNA vaccine in the cytoplasm to activate IRFs (IRF3 and/or -7) through TBK1. The TBK1-dependent signaling pathway is also important in signal transduction from the RLR families in detecting transcribed antigen mRNA. TLR3, -7 and -8 recognize RNAs in the endosome; however, the transfer of antigen mRNA into the endosome has yet to be elucidated

mediated by TANK-binding kinase 1 (TBK1) [92]. TBK1 is also known as NFκB-activating kinase (NAK). Cytoplasmic DNA is recognized by several receptors, including DAI, IFI16, DDX41 and RNA polymerase III [93]. These cytosolic DNA sensors interact with TBK1 through stimulator of IFN genes (STING), which act as adaptors in the type-I IFN production pathway [94]. This suggests that the cytosolic DNA sensors could sense a DNA vaccine and induce the antiviral responses.

In the TLR3 and RLR pathways, type-I IFN production is triggered by the phosphorylation of IRF3 and -7 by TBK1 or inducible IκB kinase epsilon (IKKε) [91, 95, 96]. IKKε (also known as IKK*i*) is encoded by the *IKBKE* gene. In mice, cells that are deficient in both TBK1 and IKKε usually fail to produce type-I IFN in response to viral infection [97, 98]. Following activation of TLRs and RLRs by viral nucleic acids, TBK1 and IKKε assemble with TNF receptor-associated factor 3 (TRAF3) and TANK to phosphorylate IRF3 and IRF7 at multiple serine and threonine residues [99–102]. The phosphorylation leads to the dimerization and nuclear transfer of IRFs, and also induces the expression of pro-inflammatory and antiviral genes [103, 104].

In general, TBK1 and IKKε are known as a related pair of non-canonical IKKs, as they differ from other IKK family members, such as IKKα and IKKβ, in that they function in the upstream of the NF-κB signal transduction cascade. [105]. Despite the structural similarities of TBK1 and IKKε, they exhibit differential gene expression patterns. Similar to IKKα and IKKβ, TBK1 is ubiquitously expressed [106]. In contrast, IKKε expression is restricted to particular lymphoid tissues, immune-related cells and various epithelial-derived cell lines [106–109].

In teleosts, TBK1 transcripts have been identified in zebrafish [89], Atlantic cod *Gadus morhua* [110], common carp *Cyprinus carpio* [73] and goldfish *Carassius auratus* [111]. The amino acid sequences corresponding to these transcripts are highly conserved with respect to TBK1s of other vertebrates [73, 112]. Studies have shown that goldfish TBK1 activates the expression of two type I IFN promoters in a process mediated by IRF3 and -7 [111] while zebrafish TBK1 is capable of interacting with TRIF, which also interacts with TLR3 [89]. In goldfish, activation of IRF3 by TBK1 also has a role in RIG-I- and MDA5-mediated signaling [111]. In fish, STING (stimulator of interferon genes) has a role similar to RLR in mammals. It stimulates type I IFN production and antiviral responses against RNA and DNA viruses. It is also called MITA (mediator of IRF3 activation). STING interacts with IPS-1 in the RLR-IFN production pathway [112]. This indicates that teleosts also possess functional TLR and RLR pathways for producing type I IFN and protecting the host against pathogenic viral infection.

From this evidence, teleost TBK1 functionally induces the type-I IFN pathway after recognition of viral nucleic acids (including dsDNAs and RNAs), involving the above-mentioned PAMPs receptors. We hypothesize a model of type-I IFN activation pathway induced by DNA vaccine. In this model, teleost TBK1 could be critically important

for immunogenicity induced by DNA vaccine, since DNA receptors (i.e., TLR9, -21 and other cytosolic DNA sensors) recognize dsDNA of a foreign plasmid vector (DNA vaccine) and RNA sensors (i.e., TLR3, -7, -8 and RLR families) probably sense antigen mRNA expressed from the DNA vaccine (Fig. 2).

Other current topics on cytosolic DNA sensors (CDSs)

In mammals, the AIM2-like receptor (ALR inflammasome) and DDX41 [DEAD/H helicase (DDX) protein] act as cytosolic DNA sensors [95, 113]. ALR is a pyrin domain-containing PRR that forms an inflammasome protein complex with an ASC adaptor to recognize viral and bacterial DNAs [113]. However, no counterparts of the AIM2 gene have been found in any fish species.

The DDX protein family comprises RNA and DNA helicases containing a DExD/H-box domain. Mammalian DDX proteins have been reported to be implicated in the regulation of gene induction and are involved in a few other processes including signal transduction pathways, mRNA splicing, and translational regulation. Several DDX proteins are thought to be involved in innate immunity because they have been shown to work as RNA sensors (e.g., RIG-I and MDA5) and signaling molecules (e.g., DDX3) [83, 114]. In mammals, myeloid dendritic cells (mDCs) have a complex of three helicases (DDX1, DDX21 and DHX36) that acts as a sensor of synthetic RNA duplex poly (I:C) in the cytosol and that pairs with the adaptor TRIF to trigger type I IFN responses [115].

Interestingly, in a cell type with limited basal IFI16 expression, DDX41 presents as an initial sensor of cytoplasmic DNA, activating type I IFN and subsequent IFI16 expression, the latter serving is an amplifier of innate immune responses [115]. In addition to the role for DDX41 with STING, DDX41 receptor directly interacts with cyclic dinucleotides (CDNs) and stimulates CDN-induced type I IFN [116]. The relative roles of STING and DDX41 in sensing CDNs are not clear. One possibility is that DDX41, instead of being the initial sensor of DNA and CDNs, may function as a signaling molecule for STING-dependent DNA and CDN responses. Recently, a central role for DDX41 in DNA-induced STING-dependent responses has been confirmed, demonstrating that in both in vitro and in vivo observations, the E3 ubiquitin ligase TRIM21 showed a negative regulator of DNA responses and that TRIM21 targeted DDX41 for degradation [117]. Because of its role in DNA sensing, DDX41, like other nucleic acid PRRs, may have a role in autoimmune regulation. DDX41has not yet been found in teleosts, although the databases show that teleosts have many types of DDX protein family genes. On the whole, the above-described DNA sensors could be related to the DNA vaccine-activated innate immune responses (shown in Fig. 2), as well.

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